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EFFECTS OF *Bacillus amyloliquefaciens* D19 CULTURE CONDITIONS ON IN-VITRO ANTIFUNGAL ACTIVITY AGAINST *Neoscytalidium dimidiatum*

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Abstract – *The fungus Neoscytalidium dimidiatum is a pathogen that causes diseases in a variety of plants, with a particular preference for dragon fruit. In the search for safe and effective control of N. dimidiatum diseases in dragon fruit, the focus has been directed towards research of biological controls. This study investigates the Bacillus amyloliquefaciens D19 culture conditions for favorable antifungal activity against N dimidiatum. The highest antifungal activity was achieved in culture maintained at 45^oC with agitation at 150 rpm for 21 hours in the defined medium comprised of 0.5 g/L $MgSO_4.7H_2O$, 3.0 g/L $NaH_2PO_4.2H_2O$, 3.0 g/L $Na_2HPO_4.12H_2O$, 10 g/L sucrose, and 10 g/L peptone with initial pH 6.0. Additionally, the antifungal activity of the bacterial culture was found to be stable at temperatures less than 60^oC and pH conditions ranging from 7.0 to 9.0. Overall, the findings of this study lay a foundation for future research into the application of biological antagonists in the control of N. dimidiatum diseases in plants.*

Keywords: antifungal, Bacillus amyloliquefaciens, culture condition, dragon fruit, Neoscytalidium dimidiatum.

I. INTRODUCTION

Neoscytalidium dimidiatum, belonging to the phylum Ascomycota, class Dothideomycetes, order Botryosphaeriales, family Botryosphaeriaceae, has been identified as the causative agent of onychomycosis and dermatomycosis in human [1] or fatal pneumonia in dolphins [2]. Furthermore, several strains of *N. dimidiatum* have been reported as a major pathogen affecting various plant hosts, including tomato, pitaya, and dragon fruit [3, 4]. Particularly, fungal infection caused by *N. dimidiatum* significantly impacts the yield and quality of dragon fruit, posing challenges for both domestic consumption and export [3]. To combat this disease and other fungal infections, chemical fungicides have been widely used in agriculture. However, their effectiveness in controlling diseases has been getting more and more limited, and their use poses risks to human health, the environment, and the development of drug-resistant fungal strains [5]. As the world moves toward eco-friendly agriculture, the adoption of biological approaches, such as antimicrobial compounds derived from microorganisms, becomes crucial. Notably, *Bacillus amyloliquefaciens*, known for its applications in manufacturing enzymes, and antibiotics, belongs to the group of Generally Recognized as Safe microorganisms (21CFR1.4) [6–8]. This species can also serve not only as probiotics in food supplements, aiming to balance the gut microbiota and promote human and animal health [9–11], but also as favorable agents for plant growth and stress tolerance [12–15]. In addition, various strains of *B. amyloliquefaciens* have been reported to exhibit antifungal activity against a number of plant pathogenic fungi such as *Colletotrichum siamense*, *Aspergillus niger*, *Aspergillus flavus*, *Mucor hiemalis*, *Fusarium oxysporum*, *Penicillium citrinum*, *Rhizopus* sp., *Trichoderma harzianum*, and *Candida albicans* [16–19]. To enhance the effectiveness of an-

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tagonistic bacteria against plant pathogens, it is crucial to investigate these bacteria and determine the optimal culture conditions for maximizing their antagonistic activity.

II. LITERATURE REVIEW

Moreover, studies have found that environmental conditions during cultivation significantly influence the synthesis of antimicrobial substances. For example, Ashwini et al. [20] reported that *Bacillus subtilis* culture exhibited antagonistic activity against *Colletotrichum gloeosporioides* OGC1, reducing anthracnose disease of chili by 65% but is not able to work after treatment at 70–100^{o}C. Song et al. [21] also demonstrated that cultural filtrate from Actinomycetes was effective against pathogenic *Colletotrichum dematium* of *Sarcandra glabra* and strain JT-2F showed the highest antagonism rate of 86.75%. In the case of *B. amyloliquefaciens* An6, culture medium supplemented with 20 g/L starch and 10 g/L yeast extract, initial neutral pH, and 30^{o}C under continuous agitation at 200 rpm resulted in a maximum bactericidal effect on *S. aureus* [22]. In addition, the strain *B. amyloliquefaciens* D19 was previously reported for its antifungal activity against *N. dimidiatum* in the study of Nguyen et al. [23]. *Bacillus licheniformis* KN12 culture suspension showed antifungal activity against *Fusarium oxysporum* and *Fusarium equiseti* in Luria-Bertani medium with an initial pH of 7.0 [24]. This study aims to investigate the impact of culture conditions on the D19 antifungal activity against *N. dimidiatum*, laying the groundwork for future sustainable practices.

III. RESEARCH METHODS

A. Microorganisms and growth conditions

The bacterial strain *Bacillus amyloliquefaciens* D19 and pathogenic fungal strain *Neoscytalidium dimidiatum* were obtained from the collection of Microbiotechnological Laboratory of the Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City. *Bacillus amyloliquefaciens* D19 was cultured in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) at 37^{o}C for 24 hours with agitation at 150 rpm, and *N. dimidiatum* was cultured on Potato Glucose Agar (PGA, 200 g potato infusion, 20.0 g/L D-glucose, 20 g/L agar, pH to 6.0) at room temperature for 3–5 days prior to be used in antagonistic tests.

B. Antifungal activity determination

The fungal antagonistic test was done using the agar well diffusion method. *Neoscytalidium dimidiatum* culture (Section IIIA) was spotted in the middle of the PGA plate and incubated at room temperature. After 24 hours, a 6-mm well was made with a cork borer at $\tilde{1}$.0 cm from the edge of the plate. A culture supernatant of *B. amyloliquefaciens* D19 (Section IIIA) was obtained by 10-minute centrifugation of the bacterial suspension at 13,000 rpm, of which 20 μL was introduced into the well. A negative control well was made using 20 μL of sterile LB broth. Fungal colony inhibition percentage (IP) was calculated after four days of incubation at room temperature as *[maximum colony diameter – inhibited colony diameter]/maximum colony diameter*.

C. Impact of initial pH, temperature, culture time, nitrogen, and carbon sources on antifungal activity of B. amyloliquefaciens D19

Bacillus amyloliquefaciens D19 grown in LB broth in shaking condition (150 rpm) at 37^{o}C for 24 hours was used as a starter (1.0% v/v) for testing different culture conditions using the agar well diffusion method. The antifungal activity of the culture supernatant in each defined condition was evaluated based on the fungal colony inhibition percentage. The experiment was done in triplicate, the data were visualized using Microsoft Excel, and ANOVA tests (α = 0.05) were done using Statgraphics Centurion 18 software.

Bacillus amyloliquefaciens D19 was grown at 37^{o}C for 24 hours in 150 rpm shaking condition in basal medium containing 0.5 g/L $MgSO_4.7H_2O$, 3.0 g/L $NaH_2PO_4.2H_2O$, 3.0 g/L $Na_2HPO_4.12H_2O$, 10 g/L D-glucose, 10 g/L peptone [25]. The antifungal activity of bacterial

culture in various initial pH conditions (4.0, 5.0, 6.0, 7.0, 8.0, 9.0±0.1) was determined by the agar well diffusion method. Briefly, basal media were adjusted to various pH values and subsequently inoculated with a 1% starter culture of *Bacillus amyloliquefaciens* D19. The bacterial cultures were incubated at 37^{o}C and 150 rpm for 24 hours. The culture supernatant was collected by centrifugation at 13,000 rpm for 10 minutes and assessed for antifungal activity using the agar well diffusion method (Section IIIB).

Subsequently, the effect of temperature and incubation time on antifungal activity was examined by culturing *B. amyloliquefaciens* D19 in basal medium with initial pH 7.0, at different temperatures 25^{o}C, 28^{o}C, 33^{o}C, 37^{o}C, 45^{o}C±0.1^{o}C. The culture supernatant was collected by centrifugation at 13,000 rpm for 10 minutes and assessed for antifungal activity using the agar well diffusion method (Section IIIB).

The effects of carbon and nitrogen sources were examined by growing the bacteria in basal media that removed the initial amount of D-glucose and peptone, respectively. Different carbon sources (D-glucose, sucrose, maltodextrin, starch, lactose) and different nitrogen sources (NH_4Cl, $NaNO_3$, NH_4NO_3, urea, peptone, yeast extract) were supplemented into *B. amyloliquefaciens* D19 culture media at 1.0% (w/v) concentration. After suitable incubation time, the culture supernatant was collected by centrifugation at 13,000 rpm for 10 minutes and assessed for antifungal activity using the agar well diffusion method (Section IIIB).

D. Effect of temperature and pH on stability of antifungal activity of the culture

The agar well diffusion method was used to study the effect of temperature and pH on the stability of antifungal activity of *B. amyloliquefaciens* D19 culture supernatant in suitable growth conditions. The effect of temperature was examined by incubating 1.0 ml of the bacterial culture supernatant for 15 minutes at different points from 60^{o}C to 95^{o}C. Effect of pH was examined by adjusting 1.0 mL of the bacterial culture supernatant to different pH levels from 1.0 to 12.0 using hydrochloric acid/potassium chloride buffer (pH 1.0–2.0), citric acid/sodium citrate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), tris amino methane/hydrochloric acid buffer (pH 8.0–9.0), sodium bicarbonate/sodium hydroxide buffer (pH 10.0), sodium phosphate dibasic/sodium hydroxide buffer (pH 11.0–12.0), followed by two hours of incubation at room temperature. Antifungal activity was evaluated based on the remained fungal colony inhibition percentage compared to the control (non-treated) by agar well diffusion method.

The experiments were done in triplicate, the data were visualized using Microsoft Excel, and ANOVA tests (α = 0.05) were done using Statgraphics Centurion 18 software.

IV. RESULTS AND DISCUSSION

A. Antifungal activity of Bacillus amyloliquefaciens D19

The antifungal activity of *B. amyloliquefaciens* D19 was first re-checked using the co-cultivation method on the PGA medium. The results depicted in Figure 1, illustrate that *B. amyloliquefaciens* D19 produces inhibitory agents against the *N. dimidiatum* strain which exhibited resistance to the antifungal agent ketoconazole (10 μg/disc). Investigations into the sensitivity to antifungal antibiotics among fungal strains isolated from diseased plants have been largely overlooked. A longitudinal study on cases of fungal infections in humans indicated that most clinical isolates of *N. dimidiatum* are susceptible to ketoconazole [26]. Nevertheless, in recent years, reports have emerged regarding numerous isolates resistant to ketoconazole [27], and even a strain resistant to both ketoconazole and itraconazole [28]. These findings underscore the emergence of *N. dimidiatum* and the significant potential of *B. amyloliquefaciens* D19 in combating pathogenic fungal infections.

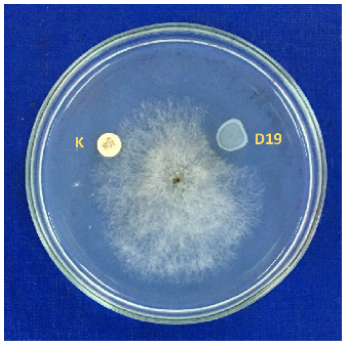

Fig. 1: Antagonistic effect of *B. amyloliquefaciens* D19 against *N. dimidiatum*

Note: K: ketoconazole

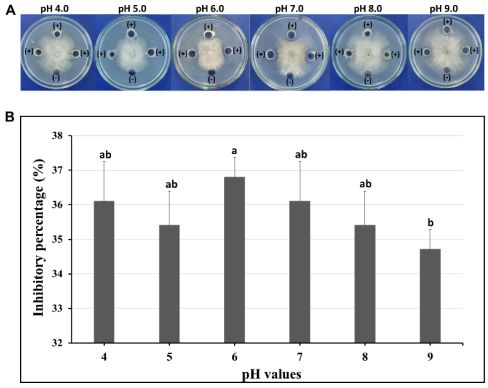

Fig. 2: Effect of initial pH on the inhibition of *N. dimidiatum* mycelial growth

Note: (+) B. amyloliquefaciens D19 culture supernatant, (-) negative control; ^{a,b} different letters indicate statistically significant differences ($p < 0.05$)

B. Effect of initial pH on the antifungal activity of Bacillus amyloliquefaciens D19 culture supernatant

After 24 hours of incubation at 37^{o}C with agitation at 150 rpm under varying initial pH conditions, the culture supernatant of *B. amyloliquefaciens* D19 was assessed for its antifungal activity. The results depicted in Figure 2A demonstrate that *B. amyloliquefaciens* D19 exhibited antifungal activity across environments with all initial pH values ranging from 4.0 to 9.0.

While the antifungal activities showed no significant differences under initial pH conditions of 4.0–8.0 (ANOVA, n = 3, 95% confidence interval) and reached the highest at pH 6.0 (36.81%±0.98%), they decreased when the bacteria were cultured at pH 9.0 (Figure 2B). As a result, the initial pH of 7.0 is selected for cultivating *B. amyloliquefaciens* D19 to produce agents antagonistic to *N. dimidiatum*. Indeed, neutral pH was found to be commonly utilized in research on the antifungal activity of different *Bacillus* species. For instance, the initial pH medium for antifungal compounds production by *B. amyloliquefaciens* C2LP and *Bacillus subtilis* SL-44 was 7.0 [29, 30]. Furthermore, these results also indicate the flexibility of the D19 strain in producing antifungal agents across a wide pH range.

C. Effect of temperature and culture time on the antifungal activity of Bacillus amyloliquefaciens D19 culture supernatant

The results depicted in Figure 3 indicate that temperature and time significantly influence the *B. amyloliquefaciens* D19 production of antifungal agents against *N. dimidiatum*. Cultures incubated at five different temperature conditions all exhibited inhibitory effects, albeit with varying activity levels depending on the temperature and incubation time. The antifungal activity at temperatures ranging from 25^{o}C to 45^{o}C tended to increase over the incubation period, peaking at 21 hours of cultivation and gradually declining thereafter (Figure 3A). There were no statistically significant differences observed in the fungal inhibitory capacity when *B. amyloliquefaciens* D19 was cultivated at temperatures between 25^{o}C and 37^{o}C for 21 hours (ANOVA, n = 3, 95% confidence interval), except at 45^{o}C where the highest activity was recorded with a corresponding inhibitory activity of 41.67%±0.62% (Figure 3B). It is worth noting that while the typical incubation time for *Bacillus* strains ranges from 30 to 48 hours, the highest antifungal activity of the D19 strain is achieved at 21 hours,

which could confer an advantage [29, 31, 32]. To meet practical and economic considerations in the application of *B. amyloliquefaciens* D19 for pathogenic fungal control, conditions of 21 hours of incubation at 37^{o}C instead of 45^{o}C were applied to subsequent experiments. Indeed, the temperature range of 28–37^{o}C was also applied in antifungal studies of various *Bacillus* strains [31, 33, 34].

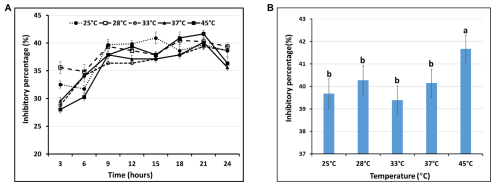

Fig. 3: Effect of temperature on the inhibition of *N. dimidiatum* mycelial growth

Note: (A) during 24 hours and (B) after 21 hours of incubation of B. amyloliquefaciens D19; ^{a,b} different letters indicate statistically significant differences ($p < 0.05$)

activity. Besides, *Bacillus* sp. BH072 also exhibited optimal antifungal lipopeptide production in a medium containing 0.98% sucrose [36].

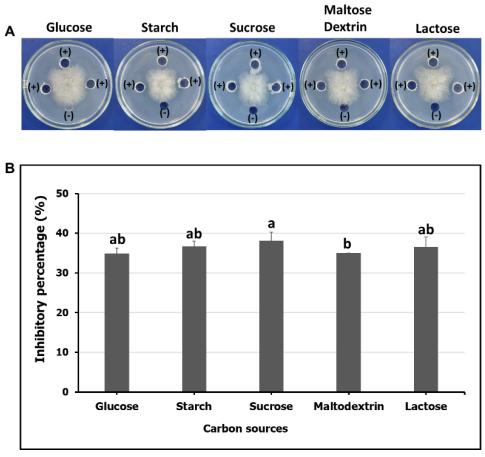

Fig. 4: Effect of carbon source on the biosynthesis of antifungal agents

Note: (-) Control, (+) B. amyloliquefaciens D19 culture supernatant; ^{a,b} different letters indicate statistically significant differences ($p < 0.05$)

D. Effect of carbon source on the antifungal activity of Bacillus amyloliquefaciens D19 culture supernatant

Figure 4 demonstrated that *B. amyloliquefaciens* D19 could utilize all five tested carbon sources to synthesize agents antagonistic to *N. dimidiatum*. The highest antifungal activity was recorded when the bacteria were cultured in a medium supplemented with sucrose (38.10%±3.89%). However, statistical analysis showed no significant differences in antifungal activity among the five carbon sources (ANOVA, n = 3, 95% confidence interval) (Figure 4B). Sucrose is inexpensive and easily obtainable, making it a cost-effective option for production. Thus, sucrose was selected as the carbon source for subsequent experiments. These findings are consistent with the study by Zhang et al. [35], which reported that *B. amyloliquefaciens*-9 cultured in an optimized medium with sucrose as the carbon source achieved the highest antimicrobial

E. Effect of nitrogen source on the antifungal activity of Bacillus amyloliquefaciens D19 culture supernatant

Both inorganic and organic nitrogen sources were shown to provide *Bacillus amyloliquefaciens* D19 with the ability to grow and produce extracellular agents that inhibit *N. dimidiatum* mycelium (Figure 5). The bacterial culture significantly inhibited the growth of fungal hyphae when grown in a medium containing yeast extract (43.94%±1.07%). The lowest inhibitory activity was observed when the bacteria were cultured in a medium containing NH_4Cl (30.30%±2.14%). Additionally, statistical analysis revealed no significant differences in inhibitory activity among the other nitrogen sources tested, except for NH_4Cl and $NaNO_3$ (ANOVA, n = 3, 95% confidence interval). In the study by Ayed [22], the *B. amyloliquefaciens* An6 strain also showed the highest mold inhibition when cultured in a basal fermentation medium supplemented with

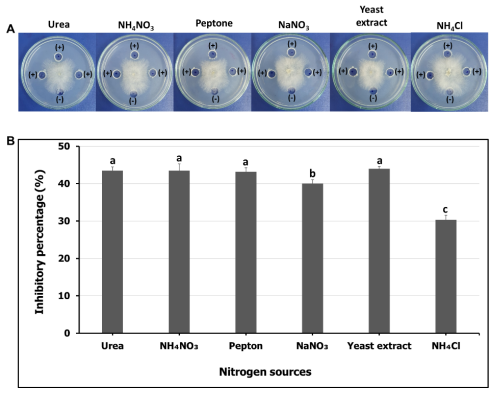

Fig. 5: Effect of nitrogen source on the biosynthesis of antifungal agents

Note: (-) Control, (+) B. amyloliquefaciens D19 culture supernatant; ^{a,b,c} different letters indicate statistically significant differences ($p < 0.05$)

yeast extract as the nitrogen source, whereas the *B. amyloliquefaciens* RC-2 strain preferred peptone to enhance its antifungal activity against *Colletotrichum dematium* [37]. Based on these results, yeast extract was selected as the nitrogen source for culturing *B. amyloliquefaciens* D19.

F. Effect of temperature and pH on the Bacillus amyloliquefaciens D19 culture supernatant

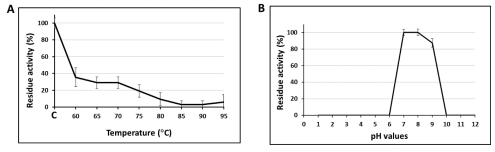

Fig. 6: Effect of temperature (A) and pH (B) on stability of antifungal agents against *N. dimidiatum* of *B. amyloliquefaciens* D19

Note: C: Control

The thermal stability and pH stability of the bacterial culture supernatant were also assessed to elucidate the nature of the antifungal agents and the preservation potential of the culture supernatant. The supernatant was subjected to various temperature conditions (60–95^{o}C) and pH levels (1.0–12.0) and the remaining antifungal activity after treatment was evaluated and presented in Figure 6. The inhibitory activity against *N. dimidiatum* mycelium decreased significantly when treated at 60^{o}C, with residual activity recorded at 35.59%±6.13% compared to the control. The activity gradually declined with increasing temperatures and was nearly lost at temperatures above 85^{o}C (Figure 6A). The heat sensitivity suggests the antifungal agents might be proteins or other heat-labile compounds. Besides, the inhibitory activity against the test mycelium remained stable in the range of pH 6.0–9.0 suggesting that the antifungal agents are most effective in slightly acidic to slightly alkaline conditions (Figure 6B). Based on the observed thermal and pH sensitivities of the culture supernatant, it can be deduced that the potential antifungal agents produced by *B. amyloliquefaciens* D19 are best suited for mild application methods and storage conditions.

V. CONCLUSION AND RECOMMENDATION

Dragon fruit is one of Vietnam's major export commodities, and the safe and effective control of the fungal pathogen *N. dimidiatum*, which causes brown spot disease, is in need of research. In this study, suitable conditions such as pH, temperature, carbon sources, and nitrogen sources for culturing the antagonistic bacterium *B. amyloliquefaciens* D19 against *N. dimidiatum* were investigated. The results demonstrated that strain D19 is highly adaptable in synthesizing antifungal agents across various carbon and nitrogen sources, a wide range of initial pH levels, and broad cultivation temperatures. The antifungal activity of the *B. amyloliquefaciens* D19 culture supernatant was unstable when exposed to elevated temperatures above 60^{o}C or extreme acidic or alkaline pH conditions. However, it is noteworthy that under practical field conditions with outdoor temperatures and diverse pH ranges, strain D19 can be directly applied and is likely to be effective. These findings suggest the potential use of *B. amyloliquefaciens* D19 for producing

biocontrol agents to manage and treat brown spot disease in dragon fruit crops.

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